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EXPERIMENTAL STUDY

Puerarin reduces apoptosis in rat hippocampal neurons cultured in high glucose medium by modulating the p38 mitogen activated protein kinase and c-Jun N-terminal kinase signaling pathways

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Abstract

OBJECTIVE: To investigate the neuroprotective effect of puerarin on rat hippocampal neurons cultured in high glucose medium, and to examine the role of the p38 mitogen activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK) signaling pathways in this effect.

METHODS: Primary cultures of hippocampal neurons were prepared from newborn Sprague Dawley rats. Neuron-specific enolase immunocytochemistry was used to identify neurons. The neurons were cultured with normal medium (control group) or with high-glucose medium (high-glucose group), and puerarin (puerarin group), a p38 MAPK inhibitor (SB239063; p38 MAPK inhibitor group) or a JNK inhibitor (SP600125; JNK inhibitor group) were added. After 72 h of treatment, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was performed to detect apoptosis, and western blotting was used to assess protein levels of p-p38, p38, p-JNK and JNK.

RESULTS: In the high-glucose group, the neuronal apoptosis rate and the p-p38/p38 and p-JNK/JNK ratios were higher than in the control group. The p38 MAPK and JNK inhibitors prevented this increase in the apoptosis rate. The apoptosis rates in the puerarin group, the p38 MAPK inhibitor group and the JNK inhibitor group were significantly decreased compared with the high-glucose group. Moreover, protein levels of p-p38 and p-JNK were significantly reduced, and the p-p38/p38 and p-JNK/JNK ratios were decreased in the puerarin group compared with the high-glucose group. In addition, compared with the high-glucose group, p-p38 levels and the p-p38/p38 ratio were reduced in the p38 MAPK inhibitor group, and p-JNK levels and the p-JNK/JNK ratio were decreased in the JNK inhibitor group.

CONCLUSION: Puerarin attenuates neuronal apoptosis induced by high glucose by reducing the phosphorylation of p38 and JNK.

Key words: Glucose; Hippocampus; Puerarin; p38 mitogen-activated protein kinases; JNK mitogen-activated protein kinases

INTRODUCTION

The incidence rate of diabetes increases every year in China, and it is a common, chronic metabolic disease

with an array of potentially serious complications such as diabetic retinitis, diabetic nephropathy and diabetic peripheral neuropathy.[1-5] As the average lifespan increases and living standards improve,[6] the central nervous system damage produced by diabetes has been attracting increasing attention.[7] The hippocampus is a critical brain region related to learning and memory in the mammalian brain, and it is more likely to be influenced by blood glucose levels.[8] Gegen (*Radix Puerariae Lobatae*) [a herb in Traditional Chinese Medicine (TCM)] is one of the most common medicines for treating diabetes. This medicine enters the meridians of the spleen and stomach, expels pathogenic factors from muscles to remove heat, generates fluid, and relieves thirst, according to the concepts of TCM. Puerarin is the main active ingredient in Gegen (*Radix Puerariae Lobatae*). In the present study, we investigated the effects of puerarin on primary rat hippocampal neurons cultured in high-glucose medium, and we examined the underlying molecular mechanisms.

MATERIALS AND METHODS

Animals

A total of 24 male and 24 female 1-day-old specific pathogen-free Sprague Dawley rats, weighing 4-6 g, were supplied by Beijing HFK Bioscience Co., Ltd. [Beijing, China. License number: SCXK (Jing) 2009-0004]. The study was approved by the Animal Welfare Ethics Committee of Peking Union Medical College Hospital.

Main reagents and instruments

The p38 MAPK inhibitor SB239063 and the JNK inhibitor SP600125 were purchased from Sigma-Aldrich (purity ≥ 98%; St. Louis, MO, USA). Puerarin was purchased from the Chinese Food and Drug Inspection Institute (purity 95.5%; Beijing, China). Sodium pyruvate, glutamine, sodium deoxycholate, dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), Tris base and poly-L-lysine were purchased from Sigma-Aldrich. p-p38, p-JNK and JNK antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). p38 antibody was purchased from Abcam (Cambridge, UK). Nerve growth factor (NGF) was purchased from Millipore (Cambridge, MA, USA). Neuron-specific enolase (NSE) antibody was purchased from Bioworld (St. Louis Park, MN, USA). HEPES was purchased from Amresco (Solon, OH, USA). In Situ Cell Death Detection Kit was purchased from Roche (Basel, Switzerland). Triton X-100 was purchased from Nacalai Tesque (Kyoto, Japan). DNase I was purchased from Takara (Shiga-ken, Japan). Phosphate buffered saline (PBS), D-Hank's, DMEM-high glucose, fetal bovine serum (FBS) and 0.25% trypsin/0.03% ethylenediaminetetraacetic acid (EDTA) were purchased from Peking Union Medical College Hospital (Beijing, China). Glucose, NaCl, KCl, Na_2HPO_4, KH_2PO_4 and $NaHCO_3$ were purchased from the Chinese Food and Drug Inspection Institute (Beijing, China). Hematoxylin and paraformaldehyde were purchased from Beijing Chemical Reagent Co., Ltd. (Beijing, China). Secondary antibody was purchased from North China Pharmaceutical (Hebei, China). Lymphocyte Separation Medium was purchased from Haoyang (Tianjin, China). Diaminobenzidine Color Developing Reagent Kit was purchased from Beijing Jinqiao Company (Beijing, China). Bicinchoninic acid protein concentration assay kit was purchased from Beyotime Biotechnology (Jiangsu, China). TCS SP2SE laser scanning confocal microscope was purchased from Leica Camera AG, (Wetzlar, Germany). CKX31SF inverted phase contrast microscope and IX71-A12FL inverted phase contrast fluorescence microscope were purchased from Olympus (Tokyo, Japan).

Preparation of stock solutions

For the puerarin stock solution, 5 mg of puerarin was dissolved in 30 ml DMEM complete medium, passed through a 0.22 μm filter, adjusted to a final concentration of 400 μmol/L, and stored at − 20 ℃. The solution was diluted to the appropriate concentration with DMEM complete medium before use.[9]

For the SB239063 stock solution, 5 mg SB239063 was dissolved in 500 μL of DMSO, and 50 μL of this stock was added to 33.931 mL of DMEM complete medium, passed through a 0.22 μm filter, diluted to a final concentration of 40 μmol/L, and stored at − 20 ℃. This solution was diluted with DMEM complete medium to a concentration of 10 μmol/L before use.[10]

For the SP600125 stock solution, 5 mg SP600125 was dissolved in 2.5 mL of DMSO, and 25 μL of this stock was added to 56.736 ml of DMEM complete medium, passed through a 0.22 μm filter, diluted to a final concentration of 40 μmol/L, and stored at − 20 ℃. This solution was diluted with DMEM complete medium to a concentration of 10 μmol/L before use.[11]

Preparation of DMEM complete medium

DMEM was supplemented with 10% FBS (final concentration), glutamine (2 mmol/L), sodium pyruvate (1 mmol/L), HEPES (20 mmol/L), 100 U/mL penicillin and 100 μg/mL streptomycin, and NGF (10 ng/mL).

Experimental groups

After 2 days of culture, the original DMEM complete medium was replaced with treatment medium. The control group was treated with DMEM complete medium. In the high-glucose group, glucose was added to the DMEM complete medium to a final concentration of 50 mM. In the puerarin group, the high-glucose medium was supplemented with 25 μmol/L puerarin. In the p38 MAPK inhibitor group, the high-glucose medium was supplemented with 10 μmol/L SB239063. In the JNK inhibitor group, the high-glucose medium

was supplemented with 10 μmol/L SP600125. All media were passed through a 0.22 μm filter and transferred to 96-well culture plates.

Isolation, purification, culturing, and identification of hippocampal neurons

The hippocampi were removed from 1-day-old Sprague Dawley rats, and the tissue was digested with trypsin. Neurons were then purified by density gradient centrifugation, and identified by NSE immunofluorescence. The neurons were observed and photographed under a confocal laser scanning microscope, using 10 randomly selected fields of view. The number of positive cells and the total number of cells in each field were counted, and the cell purity was calculated.[12]

Apoptoic cells detection by terminal deoxynucleotidyl transferasemediated dUTO nick end labeling (TUNEL)

Cells were seeded at 5×10^5 cells per well in 6-well culture plates containing a coverslip, and 3 days later, the medium was replaced with the appropriate treatment medium. Then, 72 h after culturing, the cells were rinsed and fixed with 4% paraformaldehyde at room temperature for 1 h. Cells were then permeabilized with 0.1% Triton X-100 for 6 min. For the positive control group, 50 μL of DNase I was added into the cells and incubated at room temperature for 10 min. Then, 50 μL of the TUNEL reaction mix was added, and the cells were incubated in a dark humidified chamber at 37 ℃ for 60 min. For the negative control group, only the reaction mix was added. Under the fluorescence microscope (excitation wave length of 530 nm), positive cells appeared green. Then, 50 μL of peroxidase transition agent was added, and the cells were incubated in a dark humidified chamber at 37 ℃ for 30 min. Thereafter, 50 μL of the diaminobenzidine (DAB) solution was added into the cells, and the reaction was terminated when specific yellow granules appeared in the nuclei under the microscope. After mounting, the cells were observed under the light microscope.

Western blotting for p-p38, p38, p-JNK and JNK

Cooled radio immunoprecipitation (RIPA) lysate was added to the cells, incubated on ice, and then centrifuged to obtain the supernatant. The protein concentration of each group was calculated in accordance with the Bicinchoninic acid (BCA) protein concentration kit. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto membranes. The membranes were then incubated with primary antibody (rabbit anti-mouse p-p38, p38, p-JNK or JNK polyclonal antibody) and secondary antibody (goat anti-rabbit IgG). The membranes were rinsed and photographed, and the images were then analyzed with Lab Works 4.6 image analysis software (The Lab Works, Winnipeg, Canada). Gray scale scanning values of the target proteins and the reference proteins in each group were recorded.[12]

Statistical analyses

Data are expressed as mean ± standard deviation ($\bar{x} \pm s$). All analyses were performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Before analysis, the normal distribution of the data was confirmed with a one-sample Kolmogorov-Smirnov Z test, and inter-group comparisons of independent, normally distributed samples were performed using one-way analysis of variance. The statistical significance level was set at $P < 0.05$.

RESULTS

Morphology, identification, and purity of hippocampal neurons

Newly seeded hippocampal neurons were small and round, suspended in medium, uniformly distributed, and with a good refractive index under the inverted microscope. After 1 day in culture, most cells had adhered. After 3 days, neurons, mainly consisting of multipolar cells, increased in size and were plump with halos. Most neurons were spindle or conical-shaped, and the rest were polygonal, with a greater number of (and longer) protrusions, connecting the neurons together in a sparse network. The cells cultured for 3 days were immunostained for NSE. All nuclei were stained blue, and cells with their cytoplasm and protrusions stained green were NSE-positive cells, namely neurons (Figure 1). Under the confocal laser scanning microscope, 10 fields of view were selected at random for observation. The neuronal purity, defined as the proportion of NSE-positive cells to the total number of cells, was 95.4% ± 2.3%.

Effects of puerarin, p38 MAPK inhibitor and JNK inhibitor on the apoptosis of hippocampal neurons treated with high glucose, as detected by TUNEL

Using the TUNEL assay, the nuclei of apoptotic cells were stained yellow, while surviving cells were stained blue by hematoxylin. Neurons in the control group were plump with extended axons, and their apoptosis rate was lower (control group apoptosis rate: 10.16% ± 1.83%). Neurons in the high-glucose group appeared shrunk with shorter axons, and the apoptosis rate was significantly higher (high-glucose group apoptosis rate: 28.13% ± 5.28%). The apoptosis rates in the other groups were intermediate (puerarin group apoptosis rate: 19.80% ± 3.52%; p38 MAPK inhibitor group apoptosis rate: 18.18% ± 3.97%; JNK inhibitor apop-

tosis rate: 17.41% ± 1.88%). Compared with the control group, the neuronal apoptosis rate in the high-glucose group was higher ($P < 0.01$). Compared with the high-glucose group, the neuronal apoptosis rates in the puerarin group, the p38 MAPK inhibitor group and the JNK inhibitor group were lower (puerarin group: $P < 0.05$; others $P < 0.01$). In comparison, no significant difference in apoptosis rates ($P > 0.05$) was found among the puerarin group, the p38 MAPK inhibitor group and the JNK inhibitor group (Figure 2).

Western blotting for p-p38, p38, p-JNK and JNK in hippocampal neurons

There was no significant difference in expression of the reference protein, β-actin, among the various groups. Compared with the control group, protein levels of p-p38, p38, p-JNK and JNK in the high-glucose group were increased, as well as the p-p38/p38 and p-JNK/JNK ratios. Compared with the high-glucose group, levels of p-p38 and p-JNK in the puerarin group were significantly reduced, and the p-p38/p38 and p-JNK/JNK ratios were lower. The levels of p-p38 (and the p-p38/p38 ratio) in the p38 MAPK inhibitor group were decreased, as were the levels of p-JNK (and the p-JNK/JNK ratio) in the JNK inhibitor group. Compared with the p38 MAPK inhibitor group, the upregulation of p-p38 and the increase in the p-p38/p38 ratio ($P < 0.01$) were blocked in the puerarin

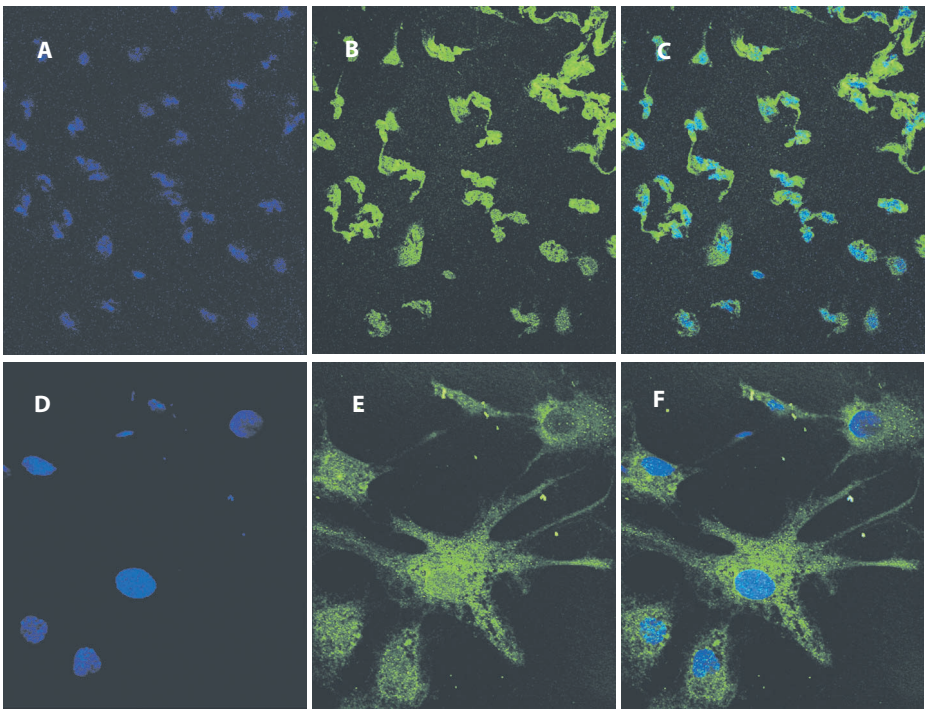

Figure 1 Purity of hippocampal neurons cultured *in vitro* as determined by immunofluorescent staining (A-C, ×200; D-E, ×600)
A, D: all the cells were stained with DAPI. B, E: neurons labeled by immunofluorescence staining for NSE. C, F: double-labeling. DAPI: 4', 6-Diamidino-2-Phenylindole; NSE: neuron-specific enolase.

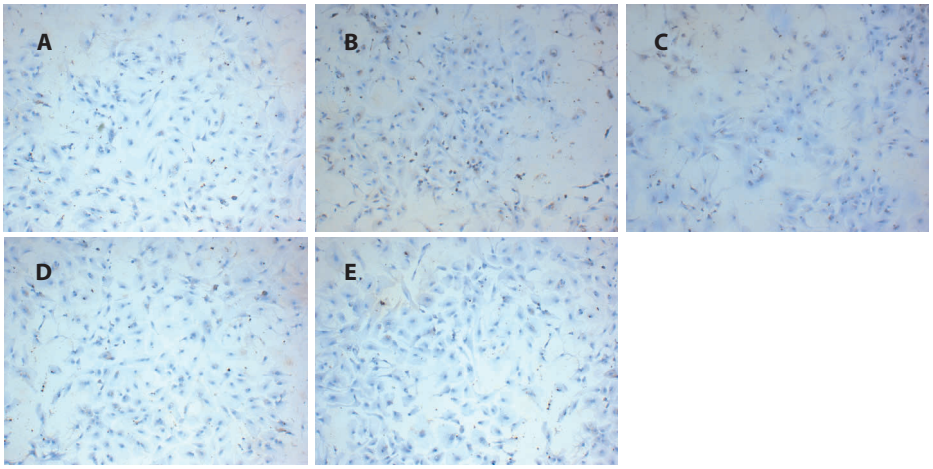

Figure 2 Apoptosis of neuron in each group was assessed using TUNEL (×200)
A: control group: cells were cultured with DMEM complete medium for 72 h. B: high-glucose group: cells were cultured with glucose (50 mmol/L) for 72 h. C: puerarin group: cells were cultured with puerarin (25 μmol/L) for 72 h. D: p38 MAPK inhibitor group: cells were cultured with SB239063 (10 μmol/L) for 72 h. E: c- JNK inhibitor group: cells were cultured with SP600125 (10 μmol/L) for 72 h. DMEM: dulbecco's modified eagle medium; TUNEL: terminal deoxynucleotidyl transferasemediated dUTO nick end labeling; MAPK: mitogen activated protein kinase; JNK: c-Jun N-terminal kinase.

group. Compared with the JNK inhibitor group, the upregulation of p-JNK (and the increase in the p-JNK/JNK ratio) ($P < 0.01$) were also prevented in the puerarin group. Gray scale scanning values for the reference protein β-actin and the target proteins p-p38, p38, p-JNK and JNK, and the p-p38/p38 and p-JNK/JNK ratios are given in Table 1. An immunoblot is shown in Figure 3.

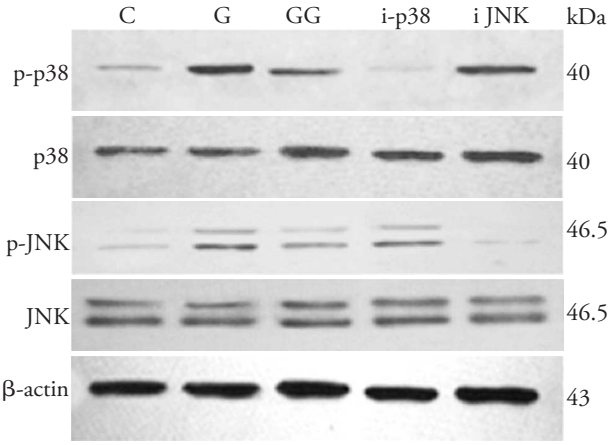

Figure 3 Western blots for p-p38, p38, p-JNK, JNK and β-actin
C: control group; G: high-glucose group; GG: puerarin group; i-p38: p38 MAPK inhibitor group; i-JNK: c- JNK inhibitor group. MAPK: mitogen activated protein kinase; JNK: c-Jun N-terminal kinase.

DISCUSSION

In recent years, diabetic cognitive dysfunction has drawn increasing attention. A series of retrospective studies have shown that the rate of cognitive decline in diabetic patients, compared with non-diabetic patients, is higher, as is the risk of cognitive dysfunction.[13] At present, the mechanisms underlying diabetic cognitive dysfunction remain unclear. However, it has been found that diabetes triggers chronic dysfunction of the central nervous system through oxidative stress,[14] inflammatory reactions,[15] increased levels of advanced glycation end products,[16] endoplasmic reticulum stress,[17] impaired mitochondrial function, and other processes.[18, 19]

The MAPK signaling pathway plays an important role in the regulation of the cell cycle and gene expression.[20] p38 MAPK and JNK/MAPK mediate apoptotic signaling after activation, and SB239063 and SP600125 are, respectively, commonly used inhibitors of these pathways. Glucose at high concentration can activate the MAPK signaling pathway in primary cultured rat dorsal root ganglion neurons, diabetic rats, and in sural nerve specimens from diabetic patients.[21]

In the present study, high-glucose medium significantly enhanced the apoptosis rate in cultured hippocampal neurons. The p-p38/p38 and p-JNK/JNK ratios were significantly increased, compared with the control group. These results suggest that the p38 MAPK and JNK signaling pathways are activated by high glucose levels. SB239063 and SP600125 blocked their respective signaling pathways and prevented the increase in the apoptosis rate, suggesting that high glucose induces apoptosis in cultured hippocampal neurons through the p38 MAPK and JNK signaling pathways. Compared with the high-glucose group, the p-p38/p38 and p-JNK/JNK ratios were both decreased in the puerarin group, suggesting that puerarin exerts its anti-apoptotic effect by reducing the phosphorylation of both p38 and JNK.

Diabetic cognitive dysfunction can be considered a combination of diabetes with dementia and amnesia according to TCM. *Shen Nong Ben Cao Jing* describes Gegen (*Radix Puerariae Lobatae*) as an herb widely used in TCM to promote circulation for stasis dispersion and to help form saliva for thirst satisfaction.[22] The present study was undertaken based on this TCM concept.

Puerarin is an isoflavone glycoside extracted from Gegen (*Radix Puerariae Lobatae*), and has been suggested to have a number of pharmacological effects, including cardiovascular protection,[23,24] anti-oxidative actions,[25] and suppression of apoptosis.[26] Current studies have in-

Table 1 Levels of p-p38, p38, phosphorylated-c--JNK) and c- JNK and the p-p38/p38 and p-JNK/JNK ratiosin the various groups ($\bar{x} \pm s$)

Group	*n*	p-p38 (/β-actin) ($\times 10^{-2}$)	p38 (/β-actin) ($\times 10^{-2}$)	p-p38/p38	p-JNK (/β-actin) ($\times 10^{-2}$)	JNK (/β-actin) ($\times 10^{-2}$)	p-JNK/JNK
Control	3	6.67±0.21	47.37±0.16	14.09±0.39	10.13±0.20	48.88±0.29	20.72±0.33
High- glucose	3	20.93±0.24^{a}	49.44±0.22^{b}	42.34±0.47^{a}	29.68±0.27^{a}	47.56±0.04^{b}	62.41±0.58^{a}
Puerarin	3	10.22±0.09^{bce}	49.96±0.17^{bf}	20.45±0.20^{aceg}	14.98±0.15^{acg}	47.53±0.10^{b}	31.52±0.39^{aeg}
p38MAPK inhibitor	3	1.62±0.37^{ac}	52.63±0.17^{ad}	3.07±0.69^{ac}	30.70±0.30^{adg}	52.16±0.10^{bcg}	58.85±0.65^{adg}
JNK inhibitor	3	18.40±0.32^{ade}	50.12±0.25b^{f}	36.72±0.47^{ace}	2.73±0.11^{ac}	47.46±0.18^{b}	5.74±0.21^{ac}

Notes: control group, cells were cultured with DMEM complete medium for 72 h; high-glucose group, cells were cultured with glucose (50 mmol/L) for 72 h; puerarin group, cells were cultured with puerarin (25 μmol/L) for 72 h; p38 MAPK inhibitor group, cells were cultured with SB239063 (10 μmol/L) for 72 h; JNK inhibitor group, cells were cultured with SP600125 (10 μmol/L) for 72 h. DMEM: dulbecco's modified eagle medium; MAPK: mitogen activated protein kinase; JNK: c-Jun N-terminal kinase. $^{a}P < 0.01$, $^{b}P < 0.05$, compared with the control group; $^{c}P < 0.01$, $^{d}P < 0.05$, compared with the high-glucose group; $^{e}P < 0.01$, $^{f}P < 0.05$, compared with the p38 MAPK inhibitor group; $^{g}P < 0.01$, compared with the JNK inhibitor group.

dicated that Puerariae can mitigate damage to PC12 cells and islet cells caused by H_2O_2,[9,27] relieve oxidative stress in hippocampal neurons induced by Aβ protein, and alleviate apoptosis in PC12 cells induced by toluene pyridine by inhibiting the JNK pathway.[9, 28-30]

In the current study, we showed that puerarin attenuates neuronal apoptosis induced by high glucose by decreasing the phosphorylation of p38 and JNK. Further study is required to clarify how puerarin reduces p38 and JNK phosphorylation.

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